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Research Article

Human MRP2 exports MC-LR but not the glutathione conjugate

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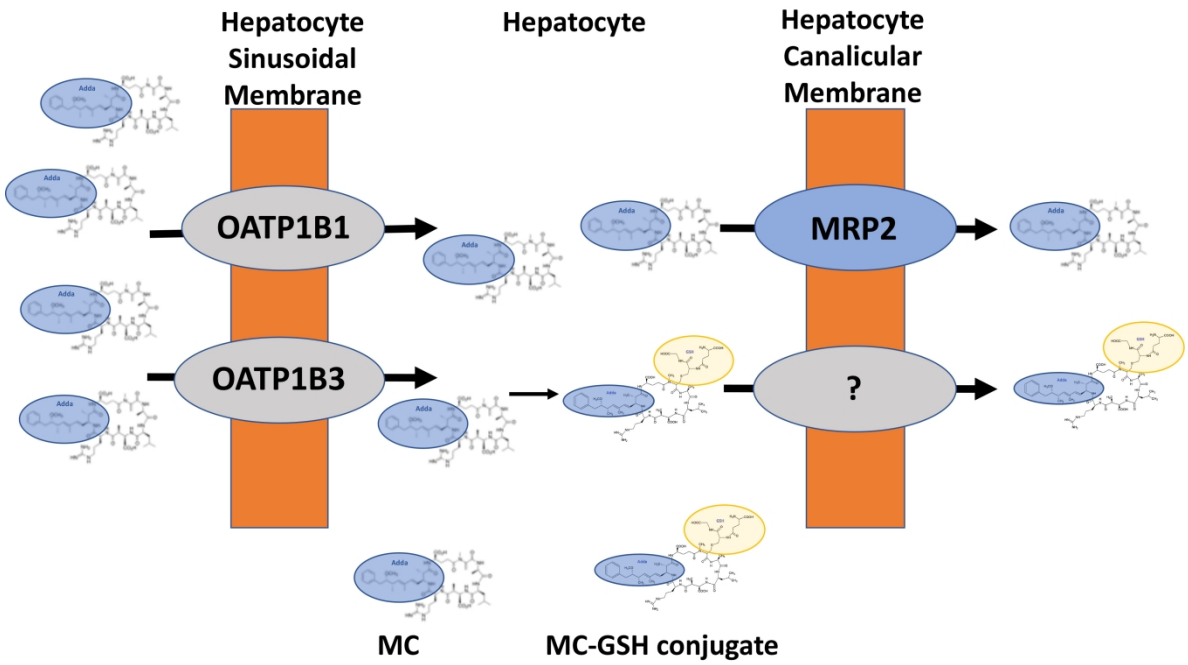
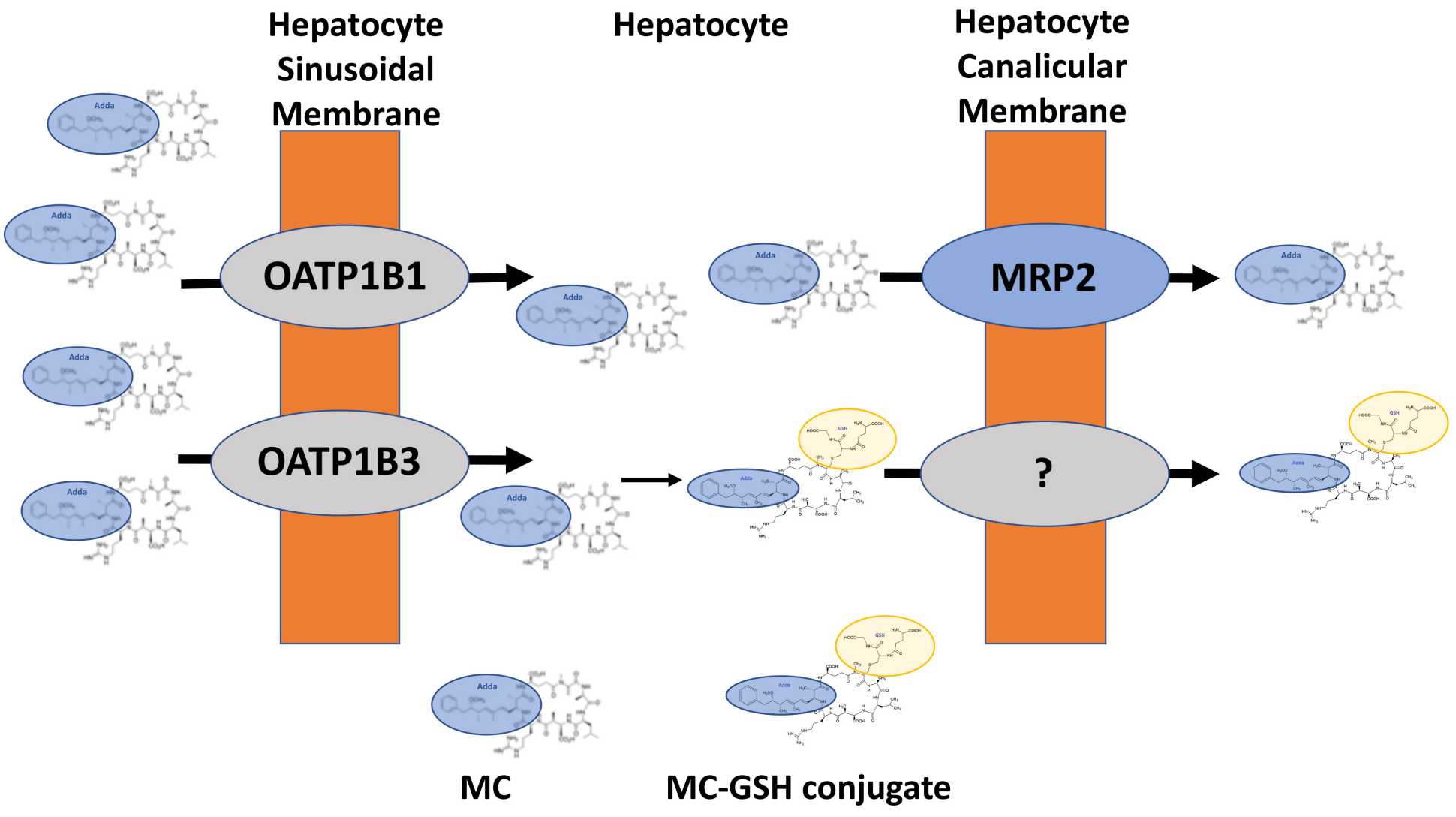
Graphical Abstract:




Hepatocyte Sinusoidal Membrane
Hepatocyte
Hepatocyte Canalicular Membrane
Adda
OATP1B1
OATP1B3
MRP2
?
GSH
MC
MC-GSH conjugate

Highlights:

- Human efflux transporters were tested for microcystin (MC) transport
- MC transport was verified via UPLC-MS/MS analyses
- Of the human efflux transporters only human MRP2 demonstrated MC transport
- MRP2 mediated MC transport was MC-congener dependent, whereby MC-LF>MC-LR>MC-RR
- No MRP2 mediated transport could be observed for the MC-glutathione conjugate

Abstract: Water contamination by cyanobacterial blooms is a worldwide health hazard to humans as well as livestock. Exposure to Microcystins (MCs), toxins produced by various cyanobacterial or blue green algae found in poorly treated drinking water or contaminated seafood such as fish or prawns are associated with hepatotoxicity, nephropathy and neurotoxicity and in extreme cases, death in humans. MC congeners, currently >240 known, differ dramatically in their uptake kinetics, i.e. their uptake via OATP1B1 and OATP1B3, in OATP overexpressing human HEK293 cells and primary human hepatocytes. It is thus likely that MC congeners will also differ with respect to the cellular efflux of the parent and conjugated congeners, e.g. via MRPs, MDRs, BCRP or BSEP. Consequently, the role and kinetics of different human efflux transporters - MRP, MDR, BCRP and BSEP in MC efflux was studied using insect membrane vesicles overexpressing transporters of interest. Of the efflux transporters investigated, MRP2 displayed MC transport. Michaelis-Menten kinetics displayed mild co-operativity and thus allosteric behavior of MRP2. MC transport by MRP2 was MC congener-specific, whereby MC-LF was transported more rapidly than MC-LR and -RR. Other human transporters (BCRP, BSEP, MRP1,3,5, MDR1) tested in this study did not exhibit interaction with MC. Although MRP2 showed specific MC transport, the MC-LR-GSH conjugate, was not transported suggesting the presence of additional mechanism(s) for the conjugate efflux.

Keywords: human MRP2; microcystin; human efflux transporters

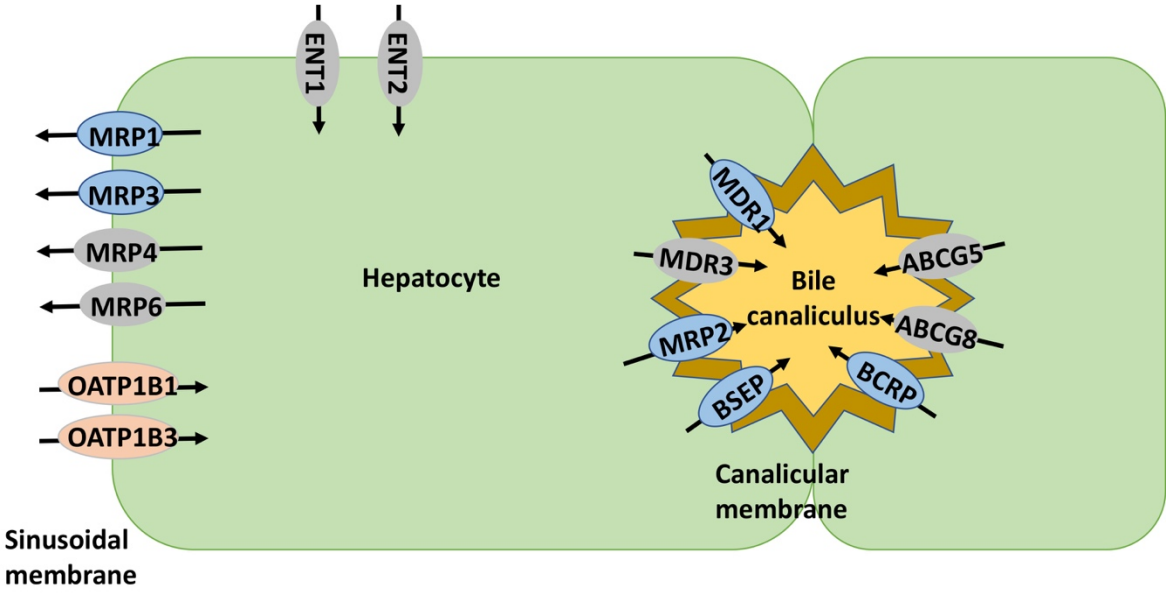
Key Contribution: We have shown that only MRP2 transports MC among the tested human efflux transporters (BCRP, BSEP, MRP1,2,3,5, and MDR1) in insect cell membrane vesicles.

Introduction

Water contamination by cyanobacterial blooms and the associated toxins, e.g. the microcystins (MCs) is a worldwide health hazard to humans. The bloom that affected almost 1000 km of the Barwon-Darling River, New South Wales, Australia, in November and December 1991 [1], the deaths of renal dialysis patients in 1996 in Caruaru, Brazil [2], or the most recent closing of the drinking water supply for the inhabitants of Toledo, Ohio, USA, resulting from recurrent *Microcystis aeruginosa* blooms in Lake Erie [3], are just a few of the many prominent examples. Exposure of humans to MC, of which more than 240 different congeners are known to date [4], have been shown to be causal in the development of hepatotoxicity and neurotoxicity and death [2]. The latter has been corroborated via a plethora of *in vitro* experiments with primary human or rodent cells [5-7] and *in vivo* exposures of rodents [8-10].

Cellular uptake of MC specifically occurs via organic anion polypeptide transporters (OATPs) [11] in humans, rodents and fish [5-7, 12, 13]. Once within the cell, MCs specifically interact with Ser/Thr protein phosphatases (PPPs) [14] as well as with other thiol containing proteins and peptides e.g. glutathione [15]. While the reversible and subsequently covalent binding to and thus inhibition of Ser/Thr protein phosphatases

[14, 16] is established to be the principle underlying mechanism of the observed cytotoxicity, the mechanisms and transporters involved in the excretion of MCs from the cells is less well understood. Indeed, despite that glutathione-conjugation of MCs was demonstrated [15, 17, 18] and the presence of MCs in the bile of trout was confirmed [19], it remains to be mechanistically proven whether unbound MC and/or conjugated MC is excreted



into the bile by a given exporter. A recent report suggested that zebrafish Abcb4, with high sequence and functional homology to mammalian ABCB1 (multi-drug resistance protein 1, MDR1), is involved in the biliary excretion of MC-LR [20]. However, questions as to the specific uptake of MC-LR in the zebrafish embryos and in Abcb4-overexpressing LLC-PK1 cells, which do not express OATP [21], as well as with regard to the detection of intra-embryonic and intra-cellular bound and free MC-LR, limit the confidence as to Abcb4 involvement in MC-LR excretion.

Figure 1. Human hepatocyte influx and efflux transporters. Known efflux transporters important for excretion/elimination of xenobiotics and drugs into bile at canalicular membrane and/or back into blood at sinusoidal membrane are shown. The human transporters: BCRP, BSEP, MRP1,2,3, and MDR1 were used in the membrane vesicle study (in blue). MRP5 was also tested albeit it is not expressed in hepatocytes. The location of MC importing transporters, OATP1B1 and OATP1B3 on the sinusoidal membrane is also indicated. Adapted from [22].

In order to provide better insight into the involvement of known human efflux transporters (Figure 1) in the excretion of unbound or conjugated MCs, we investigated the role and kinetics of different efflux transporters - MDR, MRPs, BCRP and BSEP in MC secretion using insect cell membrane vesicles specifically overexpressing ABC-transporters. Moreover, as our earlier data [5-7] strongly suggest that transport is MC congener dependent, we employed three different MC congeners (MC-LR, -LF and -RR) for the transport assays. Our present data suggest a specific role for MRP2 in transporting MCs while the other efflux transporters did not show transport of free MCs under the conditions tested. MRP2 MC efflux kinetics was congener specific, whereby MC-LF was transported at higher capacity than MC-LR or -RR.

Materials and Methods

Materials: Microcystin congeners (MC-LR, MC-LR, MC-RR) were purchased from Enzo life sciences, Loerrach, Germany. PREDIVEZ™ vesicular transport assay kits (https://www.solvobiotech.com/products/categories/predivez-vesicular-transport-reagent-kits) for human transporters, BCRP, MRP1,2,3,5 and MDR1, and membrane vesicles for BSEP (hBSEP-HEK293-VT) were procured from Solvo Biotech, Budapest, Hungary under the ReACTs program (details in Table 1). Radioactive substrates, tritium labeled N-methyl-quinidinium chloride or ^{3}H-NMQ (for MDR1) and tritium labeled taurocholic acid or taurine-2-^{3}H (for BSEP) were purchased from Biotrend, Cologne, Germany and American radiolabeled chemicals, St. Louis, USA respectively. Dimethyl sulfoxide (DMSO) (>99.98 %) was purchased from Roth. Methanol (99.8%), LC-MS grade formic acid and ammonium hydroxide were purchased from Sigma-Aldrich. All solvents for mass spectrometry were of LC-MS grade from Roth, Germany.

Vesicular transport assay: all microcystin congeners were dissolved in methanol (final solvent concentration 1%) while all inhibitors were dissolved in DMSO (final solvent concentration 1%). For the efflux transporters- BCRP, MRP1,2,3,5 and MDR1, the assay was performed essentially as given in the PREDIVEZ™ kit. The assay protocol determined the interaction of MC with the specific transporter in insect membrane vesicles using the modulation of transport of a known fluorescent/radioactive substrate. Briefly, various concentrations of MC-LR (0.41-300 µM for BCRP, MRP1,3,5 and MDR1; 0.41-1.8 mM for MRP2) or inhibitor (benzbromarone: 0.21-300 µM, omeprazole: 0.41-300 µM and verapamil: 0.41-300 µM) or solvent control (methanol or DMSO) were added to the membrane vesicle suspension (50 µg/well) in presence of AMP (12 mM) or ATP (12 mM) in 75 µl assay buffer (PREDIVEZ™ kit). For comparison of MRP2 mediated MC-LF, MC-LR and MC-RR transport, equal concentrations of MC congeners (33.3 µM) were added. The samples were incubated at 37 °C for 3-30 min (dependent on the assay, Table 1). After the reaction was completed, the membrane vesicles were collected on Millipore filter plates under vacuum and washed three times with 200 µl 1X wash buffer (PREDIVEZ™ kit). After solubilization in 100 µl detector solution (10 min, RT), the accumulated fluorescence or radioactivity was measured on a Tecan reader or scintillation counter (after adding 500 µl scintillation liquid) respectively.

For BSEP, the assay was performed as given in (Marroquin et al., 2017) with minor modifications. Briefly, various concentrations (0.41-300 µM) of MC-LR or inhibitor (glyburide: 0.013-200 µM) or solvent control (methanol or DMSO) were added to hBSEP vesicle suspension (50 µg/well) in taurine-2-H^{3} (15,000 dpm/pmol) containing assay buffer (50 mM HEPES, pH 7.5, 100 mM KNO_3,10 mM $Mg(NO_3)_2$, 50 mM sucrose, 2 µM taurocholic acid/ [taurine -2-^{3}H], 5 % DMSO) in presence of AMP (4 mM) or ATP (4 mM) and incubated for 40 min at room temperature on a shaker. Once the transport was complete, the samples were washed with 200 µl assay buffer (without radioactive substrate) and collected on Millipore 96-well filter plates and dried under vacuum. Dried vesicle samples were dissolved using 100 µl 1X detector solution (from MRP5 PREDIVEZ™ Kit) for complete dissolution of vesicles and suspended in 500 µl of scintillation liquid. Total radioactivity/well (dpm) was measured to calculate the competitive transport in presence of MC-LR.

Relative % transport in the inhibition experiments was calculated using vehicle control (methanol or DMSO) as 100% transport. Control membrane vesicles from the same source but lacking the transporter were used as confirmation of no transport in the absence of transporter. Inhibitors for fluorescent/radioactive substrate transport were used to confirm the functionality of the assays. Experimental data were evaluated by nonlinear regression analysis using GraphPad Prism 5 Software (San Diego, CA). Table 1 summarizes assay details for each transporter used in this study.

Table 1. Assay details of each tested transporter using the insect membrane vesicle approach. Probe substrate, inhibitor with incubation times are mentioned for each human transporter. Control membrane vesicles dependent on source (SF9 or HEK293) are also listed.

Transporter	Probe Substrate	Inhibitor	Control membrane vesicle	Incubation time (min)	Read out	Kit/reference

MRP1 (human)	B-GS	Benzbromarone	SB-betagal-SF9	10	Fluorescence	SB PREDIVEZ™ VT Reagent Kit for MRP1
MRP2 (human) MRP3 (human) MRP5 (human)	CDCF	Benzbromarone	SB-betagal-SF9 (MRP2, MRP3) SB-HEK293-control (MRP5)	10 30 20	Fluorescence	SB PREDIVEZ™ VT Reagent Kit for MRP2, MRP3, and MRP5
BCRP (human)	Lucifer Yellow	Omeprazole	SB-HEK293-control	30	Fluorescence	SB PREDIVEZ™ VT Reagent Kit for BCRP
MDR1 (human)	^{3}H-NMQ	Verapamil	SB-K-control	3	Radioactivity (counts per minute)	SB PREDIVEZ™ VT Reagent Kit for MDR1
BSEP (human)	Taurine-2-^{3}H	Glyburide	SB-HEK293-control	40	Radioactivity (counts per minute)	[41]

LC-MS/MS MC-LR detection: To determine MC-LR transport in MRP2 membrane vesicles, once the vesicular transport was complete and vesicles were collected on filter after washing (see above), 100 µl ice cold MeOH:H_2O (2:1) was added to the vesicle samples and incubated for 10 minutes at 2-8 °C. After vacuum filtration, these samples were collected and subjected to UPLC-MS/MS (Waters, Eschborn, Germany) to determine the accumulated MC-LR in each sample using an already developed method described in Altaner et al. [42]. Briefly, analysis of samples was performed on an ACQUITY UPLC H-class coupled to a Xevo TQ-S tandem MS (Waters). The UPLC equipped with a Waters Acquity BEH C18 column (1.6 µm, 2.1 × 50 mm) at a flow rate of 0.4 ml/min was used for detection of a 135 *m/z* daughter ion, representative of microcystin fragmentation with an LOD of 2 nM and an LOQ of 3-5 nM. Mobile phases A: 10% acetonitrile and B: 90% acetonitrile, both supplemented with 100mM formic acid and 6 mM ammonia were used. Column temperature was set to 40 °C and the injected sample volume was 5 µl. The following gradient with a total runtime of 7 min was used: the sample was loaded in 75% A, 25% B and gradient increased to 1% A, 99% B in initial 4.2 min and held for 0.5 min; the solvent composition was returned to 75% A, 25% B at 6 min and the column re-equilibrated for 1 min. Compounds entering the mass spectrometer were ionized using a capillary voltage of 3 kV, cone gas flow of 150 l/h and a nebulizer pressure of 7.0 bar. For dissolution, a nitrogen flow of 1000 l/h at 500 °C was used. Once the MC-LR levels (ng/ml) were obtained using TargetLynx software (Waters), kinetic parameters by fitting calculated MC-LR transport values (nmol/mg/min) to the Michaelis–Menten equation by nonlinear regression analysis were generated. The data was further transformed for analysis with the Hanes–Woolf plots using Graph Pad Prism 5.

MC-LR-GSH synthesis: Semi-preparative HPLC was performed with a LC-20A prominence system by Shimadzu (degasser: DGU-20A3, pumps: LC-20AT, autosampler: SIL-20A, communication module: CBM-20A, diode array detector: SPD-M20A (D2 lamp), column oven: CTO-20AC) with a Vydac® Protein and Peptide C18 4.6 x 250 mm, 5 µM column by Grace (flow rate 0.9 ml/min). Analytical LC-MS was performed with a LCMS2020 by Shimadzu (pumps: LC-20 AD, auto sampler: SIL-20A HT, UV-Vis detector: SPD-20A, oven: CTO-20AC, communications bus module: CBM-20A, ESI detector, software LCMS-Solution) with a Kinetex® 2.6 µM C18 100 Å, 150 x 4.6 mm column by phenomenex (flow rate 0.4 ml/min). For both LC devices a binary gradient of acetonitrile (Solvent B) in water (Solvent A) supplemented with 0.1% formic acid was used. High resolution mass spectrometry (HRMS) was measured on a micrOTOF II from Bruker and analyzed with Xcalibur v3.0 by Thermo Fischer Scientific.

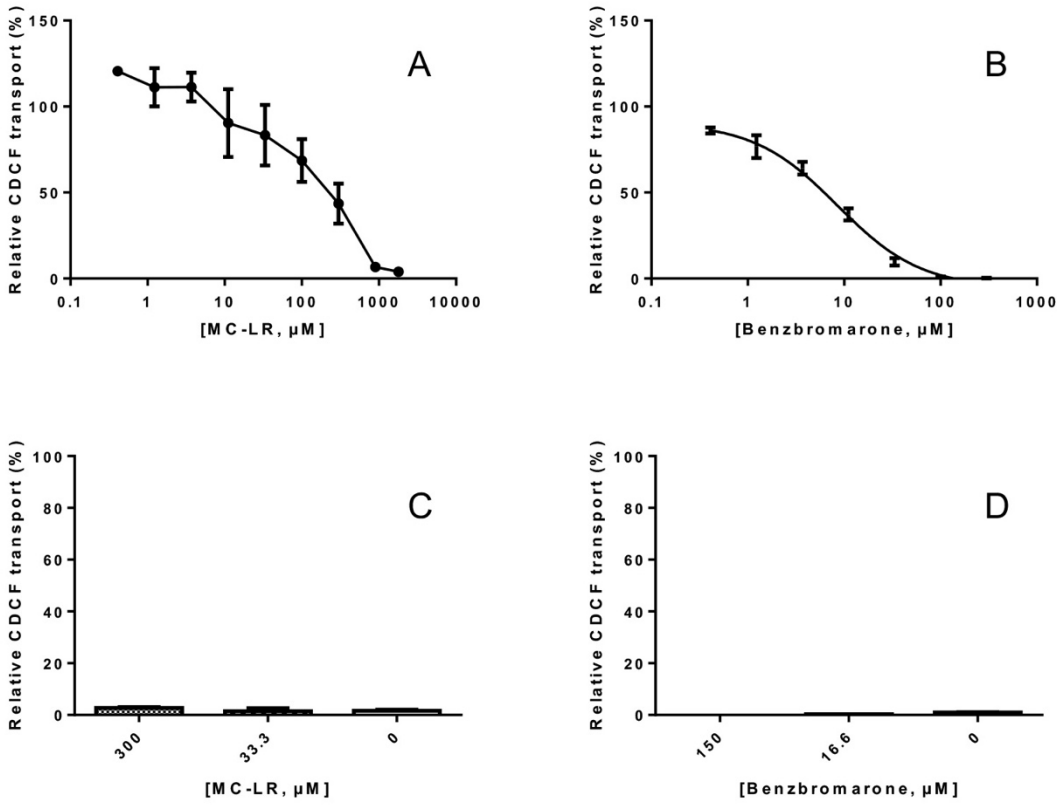
MC-LR (0.5 mg, 0.5 µmol) was dissolved in methanol (200 µl) and glutathione (7.4 mg, 24.1 µmol) in water (210 µl) was added. After addition of 1 M KOH (42 µl), the reaction mixture was incubated at 40 °C for 1 h under gentle mixing. The crude product was purified by HPLC (gradient: 30-100% B in 15 min) and the MC-LR-GSH adduct (R_t= 10.0 min, 350 µg, 0.27 µmol, 54%) was obtained as a white solid. LC-MS analysis (gradient: 30-75% B in 15 min) of the purified product (R_t= 7.2 min) gave a single peak with the correct mass (Suppl. Fig 2). HRMS *m*/*z* calculated for $C_{59}H_{93}N_{13}O_{18}S^{2+}$: 651.8236 [M+2H$^+$]; found: 651.8237.

Data analysis: For all figures, mean ± SEM is shown for at least 3 true replicates unless otherwise specified. For statistical analysis of Figure 4, ANOVA with Bonferroni correction and an additional t-test was used for pairwise comparisons.

Results

We [19] and others [23-25] have reported the presence of unconjugated MCs in bile and urine respectively of trout, carp and pig. To establish which efflux transporter could be involved in a possible biliary secretion of MCs in humans, we utilized insect cell vesicles over-expressing eight human ABC-transporters to assess possible transport of MCs. These membrane vesicles consisted of total cell membrane fractions forming vesicles but lacking, nucleosolic and cytosolic components. As the orientation of the vesicles is random, part of the efflux transporters of interest are present in an inside-out configuration. The consequence of the latter is the inversion of the original direction of transport of the efflux transporter and absence of intracellular components potentially interacting with the transported substrate of interest and thus resulting in the accumulation of a known transporter substrate in the lumen (inside) of the membrane vesicle. Interaction with the transporter could then be quantified using either fluorescent or radioactive labelled standard substrates. Thus, if a given MC congener is a substrate of a specific ABC-transporter it should compete with its fluorescent or radioactively labelled substrate thereby reducing the rate of the labelled substrate transport and intra vesicular lumen accumulation.

Based on our previous study [19] in trout, a specific transport mechanism for excretion into bile was suggested, we thus tested whether *a priori* MRP2, localized at the bile canalicular membrane (Figure 1) is involved in MC efflux, albeit knowing that other transporters e.g. MDR1 could be responsible as well [26]. For this purpose, we incubated MRP2 insect membrane vesicles in the presence of the prototypical MRP2 substrate CDCF with increasing MC-LR concentrations in presence of ATP or AMP. Obviously with this type of experimental set-



up we cannot make any inference on the type of transport inhibition, i.e. whether this results from mere binding to the transporter or resulted from competitive interaction at the substrate binding site. As expected, a decreased CDCF transport was observed with increasing MC-LR concentration (Figure 2A). Decreased CDCF accumulation in MRP2 membrane vesicles was also observed with increasing concentrations of benzbromarone (Figure 2B), an inhibitor of MRP2 [27] (Table 1). In contrast, in the absence of MRP2 expression, no CDCF transport was observed irrespective of whether or not MC-LR and benzbromarone were present (Figure 2C-D). The latter data confirmed the functionality of the MRP2 expressed in the membrane vesicles, excluded passive entry of CDCF by diffusion into the vesicular lumen and make an unspecific membrane alteration by MC-LR very unlikely. Moreover, these data demonstrated MC-LR concentration-dependent modulation of CDCF transport, thereby corroborating the initial assumption that MC-LR, *de minimis*, binds to MRP2.

Figure 2. MRP2-mediated CDCF transport in the presence of MC-LR in insect membrane vesicles. A. In an indirect vesicular transport assay approach, decrease in CDCF fluorescence was observed with increasing MC-LR concentration in MRP2- membrane vesicle fractions. B. The MRP2 inhibiting benzbromarone [27] was used as a control to demonstrate CDCF transport inhibition. Membrane vesicle samples without transporters were used as negative control for MC-LR (C) and benzbromarone transport (D). The absolute Relative Fluorescence Units (RFU) for 100% CDCF transport were 36701 ± 2852. Shown are n= 3 true replicates, mean ± SEM.

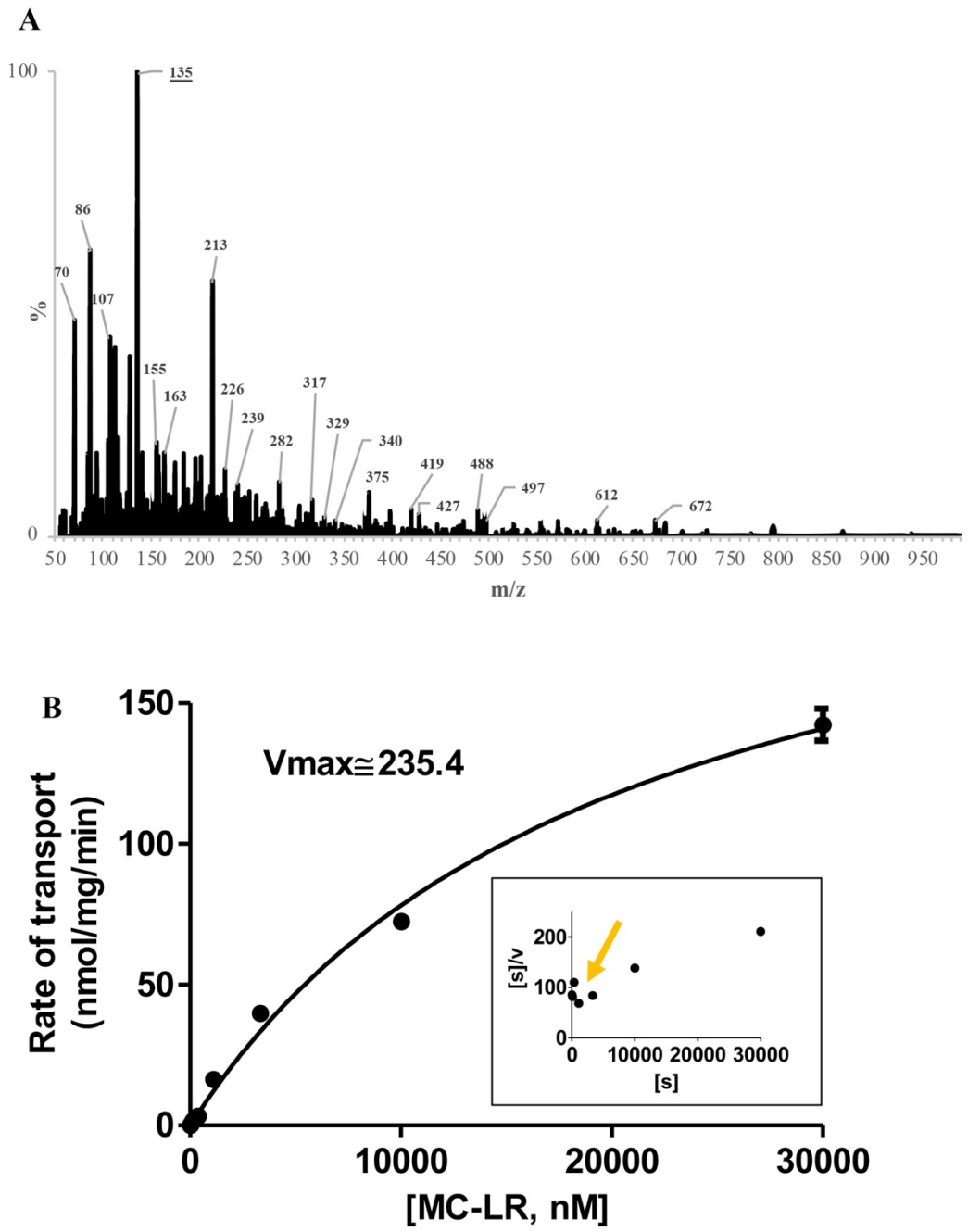


Figure 3. LC-MS/MS detection of MC-LR uptake in MRP2-membrane vesicles and assessment of Michaelis-Menten kinetics. Vesicular lumen content of MRP2-membrane vesicles treated with different concentrations of MC-LR were subjected to LC-MS/MS analysis. A: representative LC-MS/MS full scan of MC-LR ($M+H^+$=995) with daughter ion at 135 *m/z*, used for detection. B. Michaelis-Menten kinetics of MC-LR concentrations within the vesicular lumen, transformed to a Hanes-Woolf plot (inset), displaying mild cooperativity (arrow). Shown are n= 3 true replicates; mean ± SEM.

To confirm that MC-LR is actually transported by MRP2, the MC-LR lumen concentration of MRP2 membrane vesicles exposed to increasing MC-LR concentrations were analyzed using LC-MS/MS (Figure 3A) and the analytical data evaluated using Michaelis-Menten kinetics (Figure 3B). The rate of MC-LR transport

increased with MC-LR concentrations. V_{max} was approximated to 235 nmol MC-LR /mg prot x min. Transformation of the data using a Hanes-Woolf plot (Figure 3B inset) suggested mild cooperativity [28] and thus an allosteric behavior of MRP2, as was also shown previously for other low-molecular weight MRP2 substrates [29]. This precludes an exact calculation of the k_m value. It has previously been postulated [30, 31] that the binding site of MRP2 may be rather large and in case of small substrates, two substrate molecules (here, MC-LR) may bind sequentially to or close to the binding site, leading to an allosteric behavior of MRP2, visible as the curvature (marked by the arrow) on the plot.

To assess whether MC congener structure variability has an impact on MRP2 transport, we treated MRP2 membrane vesicles in the above assay with equal amounts (33.3 µM) of various MC congeners- MC-LF, MC-LR and MC-RR and assessed the inhibition of CDCF transport (Figure 4). CDCF transport activity was significantly decreased for MC-LF compared to the more hydrophilic MC-LR and MC-RR, thus suggesting a higher affinity for MC-LF than for MC-LR or -RR. The latter finding concurs with similar MC congener preferences reported for OATP-mediated cellular uptake [7].

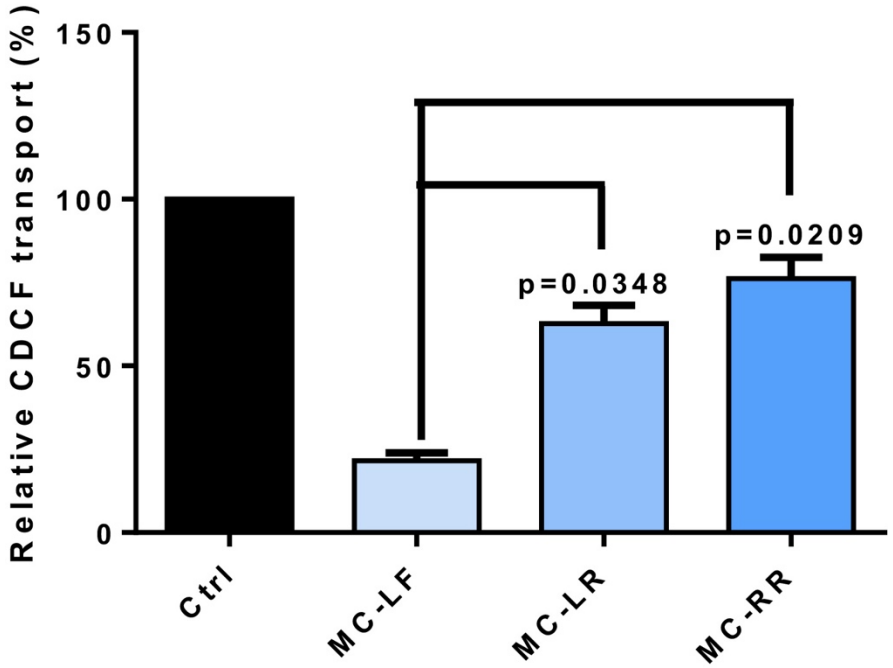


Figure 4. MC-LF is transported more efficiently than MC-LR and MC-RR. 33.3 µM MC-LR, MC-RR or MC-LF were added to MRP2-membrane vesicles and competitive CDCF transport was determined after 30 min. 100% CDCF transport is achieved in presence of solvent only. The absolute RFU value for 100% CDCF transport: 37902 ± 1725. Shown are n= 3 true replicates, mean ± SEM. $p<0.05$; ANOVA with Bonferroni correction and an additional t-test was used for pairwise comparisons.

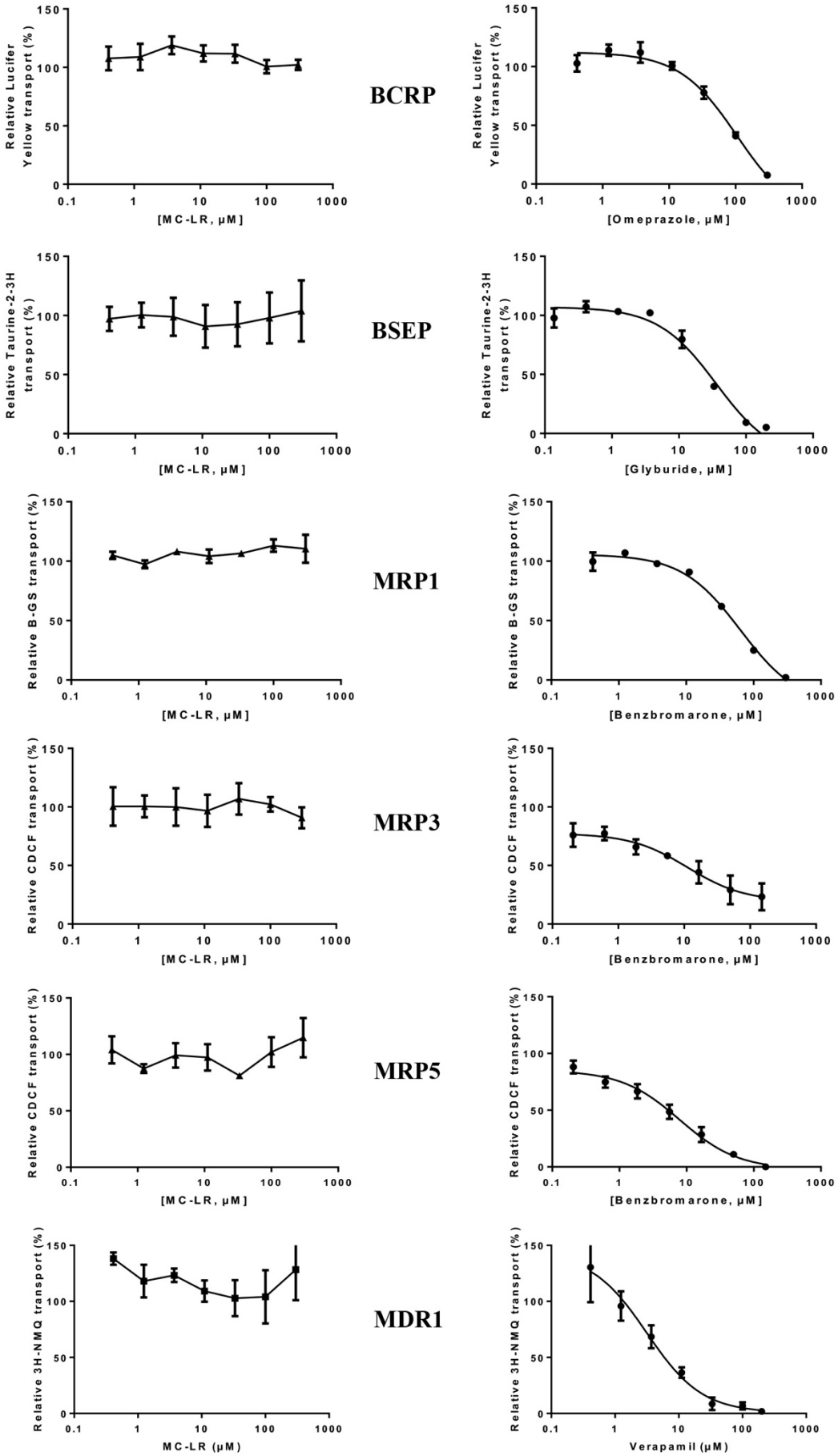


Figure 5. Efflux transporters: BCRP, BSEP, MRP1, MRP3, MRP5, and MDR1 do not transport MC-

LR. Neither BCRP, BSEP, MRP1, MRP3, MRP5 or MDR1 presented with an appreciable MC-LR transport (left panel), despite that principally functional transport was demonstrated using the respective transporter inhibitors (right panel). % Transport was calculated with vehicle control (methanol or DMSO) resulting in 100% Fluorescent/radioactive substrate transport. The absolute RFU value for 100% Lucifer Yellow transport (BCRP): 30781 ± 1588, Taurine-2-^{3}H transport in CPM (BSEP): 29332 ± 435, B-GS transport (MRP1): 25947 ± 1465, CDCF transport (MRP3): 28844 ± 1483, CDCF transport (MRP5): 31126 ±6801, ^{3}H-NMQ transport in CPM (MDR1): 2071 ± 389. Shown are n= 3 true replicates, mean ± SEM.

In addition, we tested whether other human ABC-transporters - BCRP, BSEP, MRP1, MRP3, MRP5 and MDR1 show transport of MC-LR in membrane vesicles (Figure 5). While use of the respective transporter's inhibitors demonstrated the functionality of the expressed transporter assays (Figure 5 right panel), exposure to MC-LR did not result in a concentration dependent reduction of reporter substrates (Figure 5 left panel), thereby suggesting that these transporters are not involved in MC-LR transport. Control membranes (without transporter) treated with MC-LR were used as control for transport specificity and showed no MC-LR transport in the absence of the corresponding transporter (Suppl. Fig 1). MRP4 could not be tested due to vesicle leakage and lack of significant differences between signal and background noise. Control membranes, lacking expression of MRP4 did not demonstrate increased reporter substrate or MC-LR levels thereby precluding presence of passive diffusion of either of the compounds (data not shown).

MCs were suggested to be exported from hepatocytes as conjugates (GSH and CYS) in rodents [32] as well as fish [19] although the transporter involved in their efflux is currently not established [33]. In order to test whether MRP2 transports the MC-LR glutathione conjugate (MC-LR-GSH), MC-LR-GSH was synthetized by reacting MC-LR with an excess of glutathione under basic conditions and purification of the conjugate by HPLC [34, 35]. As shown in Figure 6, transport of MC-LR-GSH by MRP2 is not suggested, at least under the conditions of the current setting. Due to limitations of available insect membrane vesicles containing the other exporters, we were unable to test whether any of the other transporters could be involved in the export of MC-LR-GSH.

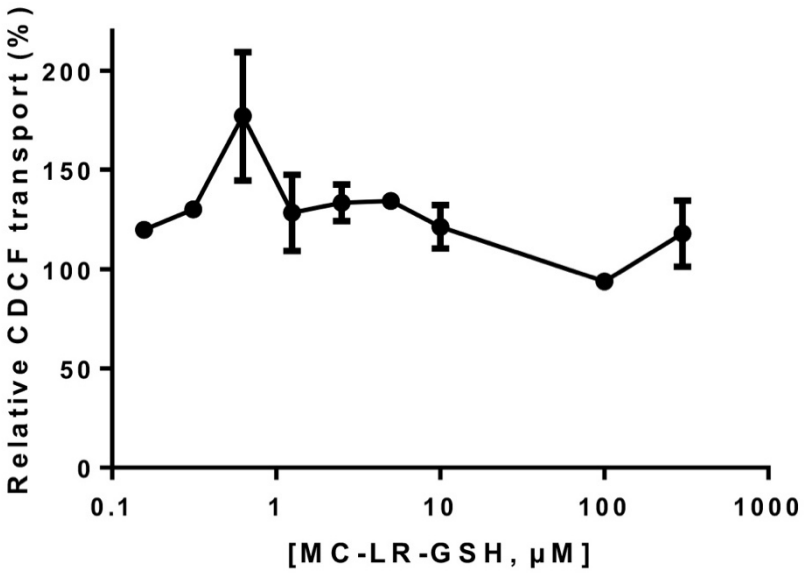


Figure 6. MRP2 does not transport MC-LR-GSH. Competitive CDCF transport was calculated after exposure of MRP2 membrane vesicles to different MC-LR-GSH concentrations (0-300 µM). % Transport was calculated with vehicle control (water) resulting in 100% Fluorescent substrate transport. The RFU for 100% CDCF transport were 13272 ± 1823. Shown are n= 3 true replicates, mean ± SEM.

Discussion

Microcystins taken up into hepatocytes via OATP1B1 and -1B3 are rapidly interacting with intracellular thiol containing peptides, polypeptides and macromolecules. The reversible and covalent interaction [16] with the catalytic subunit of Ser/Thr protein phosphatases (PPPs) [14] occurs in competition with glutathione conjugation [32] and the efflux from hepatocytes either as unchanged parent compound or as the glutathione conjugate. Although PPP inhibition, and thus the onset of cytotoxicity, is predominated by the interaction of unchanged parent MC, it was reported that glutathione conjugates as well as MC-cysteine conjugates, resulting from metabolism via γ-glutamyl transpeptidase and membrane bound dipeptidase [36], can also be toxic, albeit much higher concentrations are required for a similar degree of inhibition achieved with the parent MC-LR or MC-YR [37]. As "toxicity" is primarily the result of PPP inhibition, it can be safely assumed that the observed MC-cysteine and MC-glutathione conjugate toxicity in *i.v.* exposed mice, is a function of their inherent PPP inhibition capability. Although it remains to be demonstrated how either of the MC-cysteine or the MC-glutathione conjugates would fit within the hydrophobic groove of the PPP catalytic subunit [38], the current toxicity data do suggest that glutathione conjugation is not entirely a detoxification reaction and thus is not sufficiently protective. Moreover, analytical assessment of MC metabolites murine, rat and porcine livers suggest that MC-glutathione conjugates can be further metabolized to MC-cysteine conjugates within the liver [15, 37, 39]. The latter emphasizes the importance of hepatocyte efflux transporters in reducing the cellular load of MC parent compound and metabolites.

It was surprising, however, that of the efflux transporters tested with the insect cell membrane vesicle assay (Figure 5), only MRP2 demonstrated appreciable MC-congener transport (Figures 2 and 3). Moreover, and contrary to our expectations, MRP2 did not transport the corresponding MC-LR-glutathione conjugate (Figure 6), indicating that other transporters are responsible for the efflux of MC-cysteine or the MC-glutathione conjugates from hepatocytes into the bile [19, 24, 25, 39] or possibly even back into the sinusoidal blood [40]. Some of the potential glutathione-conjugate transporters that indeed could transport MC-glutathione conjugates back into the sinusoidal blood would be MRPs 1,3, 4, and 6 (Figure 1). However only additional experiments using glutathione conjugates of a number of MC congeners and different ABC-transporter expressing systems, would allow better judgment of the situation. As MC-congener transport by MRP2 demonstrated similar MC-congener dependency as we have previously demonstrated for the OATP uptake transporters [7], the question remains as to whether MC influx (OATP) and efflux (MRP2) rates compare. Indeed, we have established that for MC-LR influx via OATP1A2, 1B1 and 1B3 expressed in Xenopus oocytes, the v_{max} were 6, 3 and 11 fmol/oocyte·min, while the k_m values were 20±8, 7±3, and 9±3 µM, thereby demonstrating dramatically differing capacities despite similar k_m values [12]. Indeed, OATP1B1 and 1B3 appeared to have nearly double the affinity for MC-LR than OATP1A2, while the capacity for transporting MC-LR of OATP1A2 remained somewhere between OATP1B1 and 1B3. Although difficult to compare, as we didn't employ Xenopus oocytes as the transport testing system as was used for the OATPs, the approximated v_{max} and k_m values obtained for MRP2 in insect membrane vesicles were 235 nM/mg·min and 21 µM, thus suggesting that the affinity for MC-LR of MRP2 is comparable to that of MC-LR to OATP1B1 and 1B3. In view of the fact that it was unclear how much OATP was expressed in the Xenopus oocyte system and how much MRP2 was present in the insect membrane vesicles, the hugely different transport capacity may be misleading. Irrespective of the latter the results we present here strongly suggest that MRP2 is a low affinity high capacity transporter for MC congeners albeit not of their glutathione conjugates. As most bile analyses for MCs and their conjugates were carried out via functional (PPP inhibition) but not via analytical (LC-MS/MS) means [19], it may well be that only the MC congener parent compound but not the glutathione or cysteine conjugates are excreted into the bile, whereas the glutathione or cysteine conjugate are excreted back into the sinusoidal blood. Although at present the latter is but a mere hypothesis, positive proof would have major implications on the evaluation of MC congener kinetics in humans and thus human risk assessment of MCs.

Author Contributions: G.K. and D.R.D. conceived the study. G.K. designed, performed, and analyzed all the experiments. R.F. with input from V.W., synthesized and analyzed the purity of the MC-LR-GSH conjugate used in Fig 6. G.K. and D.R.D. wrote the manuscript with input from all authors. All authors critically evaluated the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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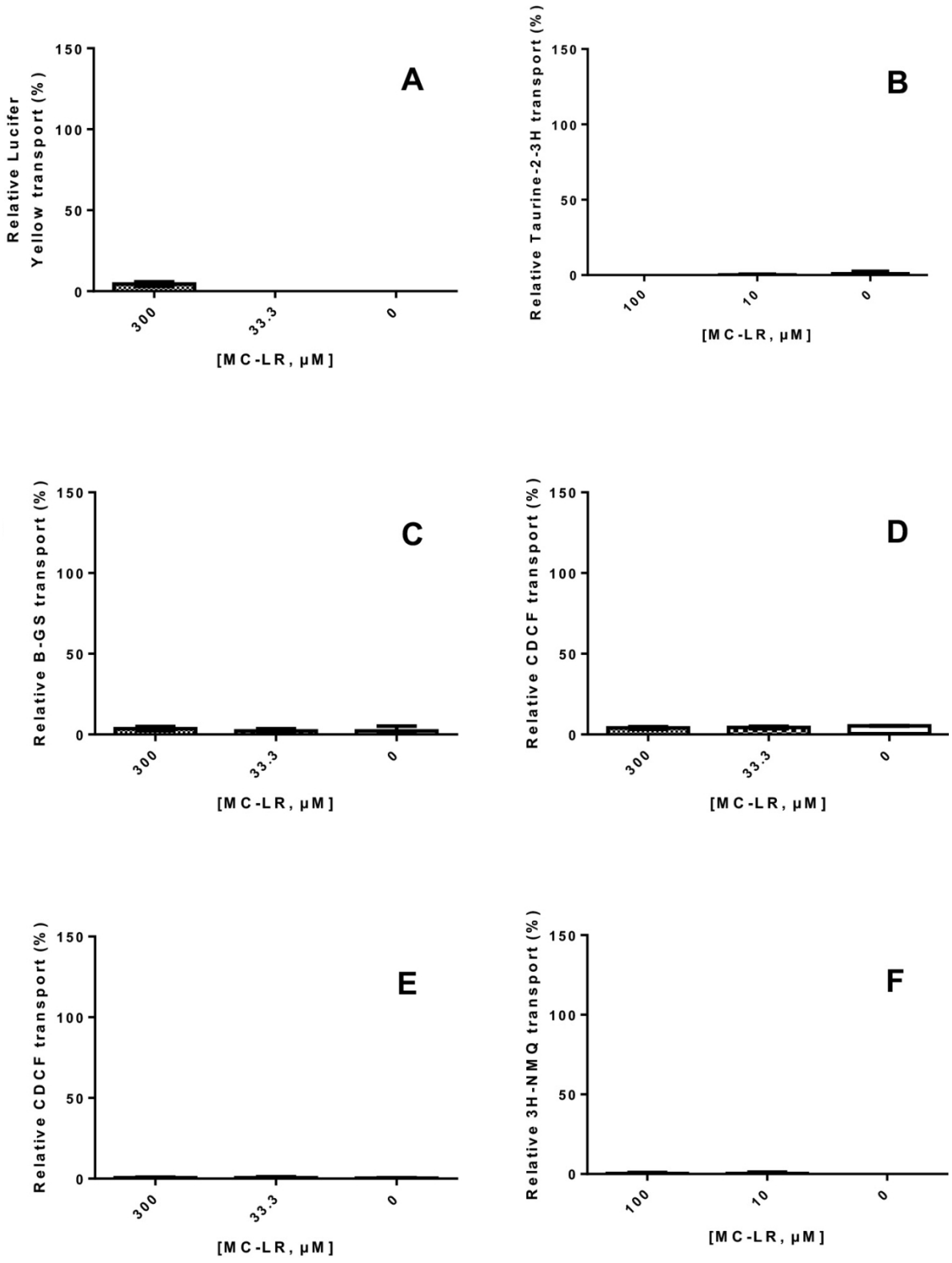
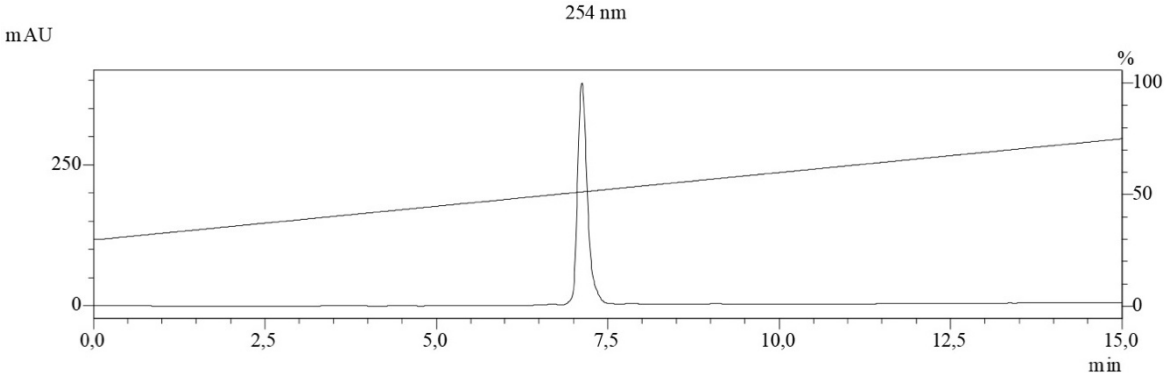
388 **Supplementary Materials:**

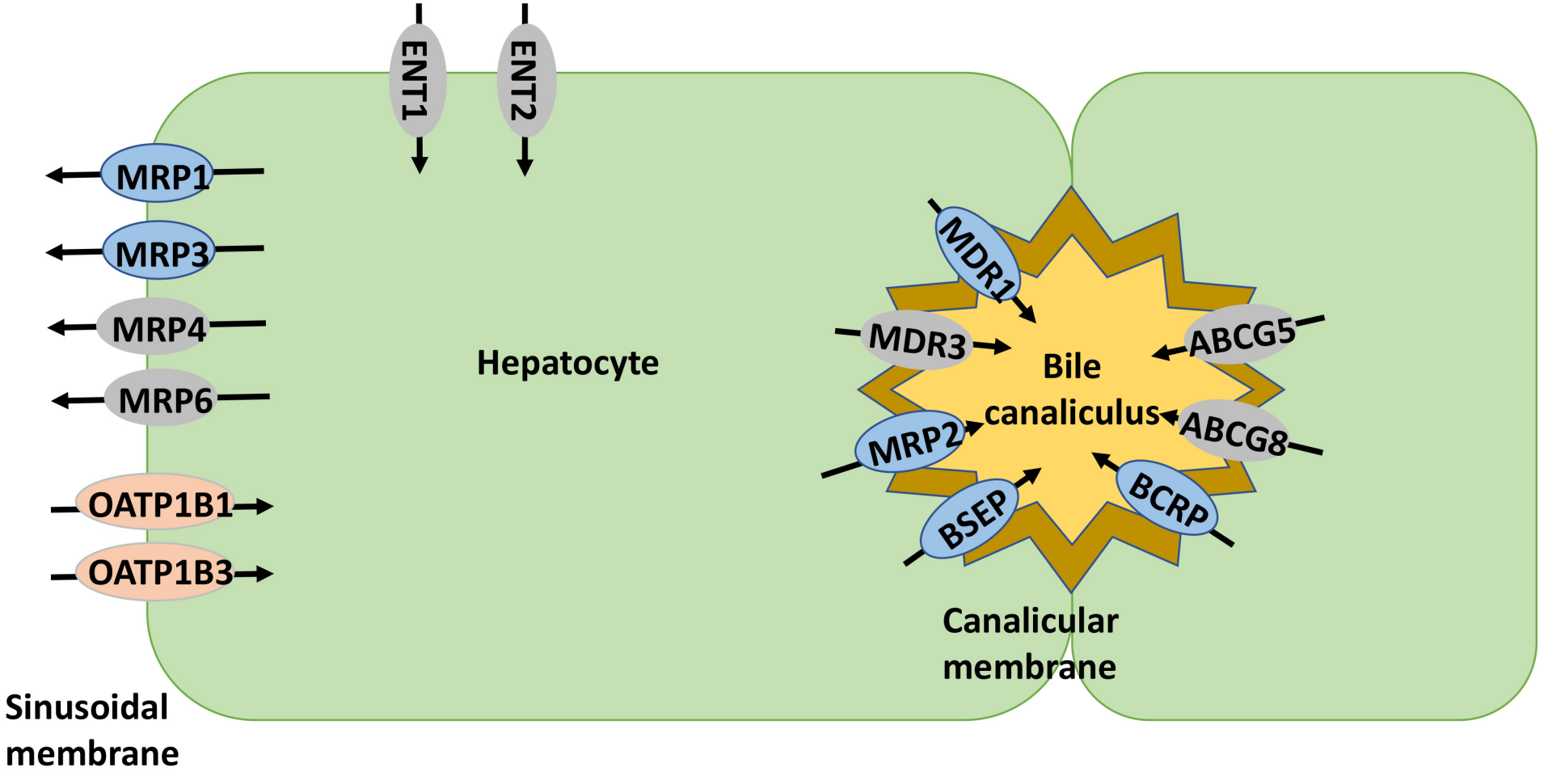


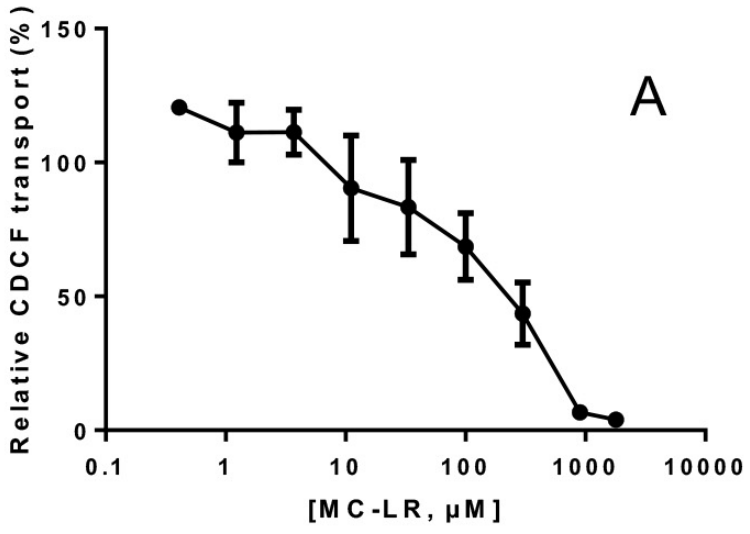
389 **Supplementary Figure 1. Membrane vesicles without efflux transporters: BCRP, BSEP, MRP1, MRP3,**
390 **MRP5, and MDR1 do not transport MC-LR.** Control experiments with membrane vesicles lacking efflux
391 transporters (BCRP (1A), BSEP (1B), MRP1 (1C), MRP3 (1D), MRP5 (1E) and MDR1 (1F)) did not show
392 MC-LR transport. % Transport was calculated with vehicle control (methanol or DMSO) resulting in 100%
393 Fluorescent/radioactive substrate transport. For 100% Lucifer Yellow transport (BCRP) amounted to 30781 ±
394 1588 RFU. For taurine-2-^{3}H (BSEP) 100% transport amounted to 29332 ± 435 CPM, For B-GS transport

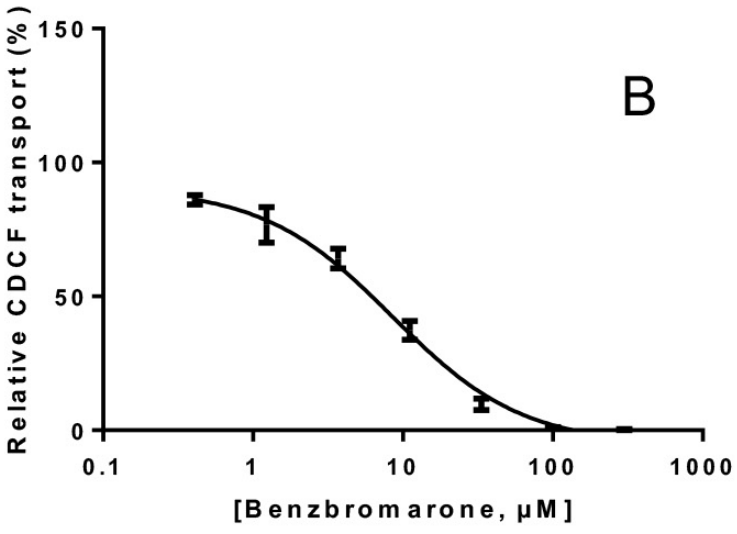
(MRP1) 100% transport amounted 25947 ± 1465 CPM. For CDCF transport (MRP3 and MRP5), 100% transport amounted to 28844 ± 1483 RFU and 31126 ±6801 RFU. For ^{3}H-NMQ transport (MDR1) 100% transport amounted to 2071 ± 389 CPM. Shown are n= 3 true replicates, mean ± SEM.

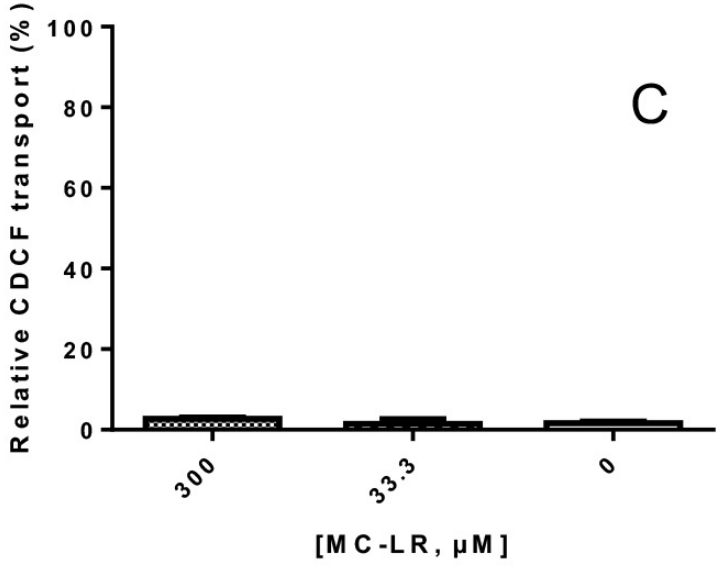
Supplementary Figure 2. LC-MS chromatogram (254 nm) of MC-LR-GSH conjugate after HPLC purification showing a single product peak (R_t= 7.2 min).

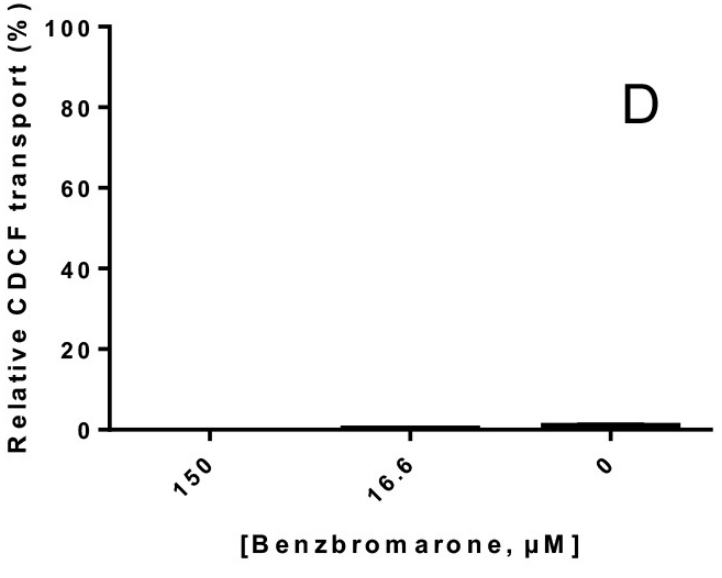



ENT1
ENT2
MRP1
MRP3
MRP4
MRP6
OATP1B1
OATP1B3
Hepatocyte
MDR1
MDR3
ABCG5
Bile
canaliculus
MRP2
ABCG8
BSEP
BCRP
Canalicular
membrane
Sinusoidal
membrane


A
Relative CDCF transport (%)
150
100
50
0
0.1
1
10
100
1000
10000
[MC-LR, µM]


B
Relative CDCF transport (%)
150
100
50
0
0.1
1
10
100
1000
[Benzbromarone, µM]


C
Relative CDCF transport (%)
100
80
60
40
20
0
300
33.3
0
[MC-LR, µM]


D
Relative CDCF transport (%)
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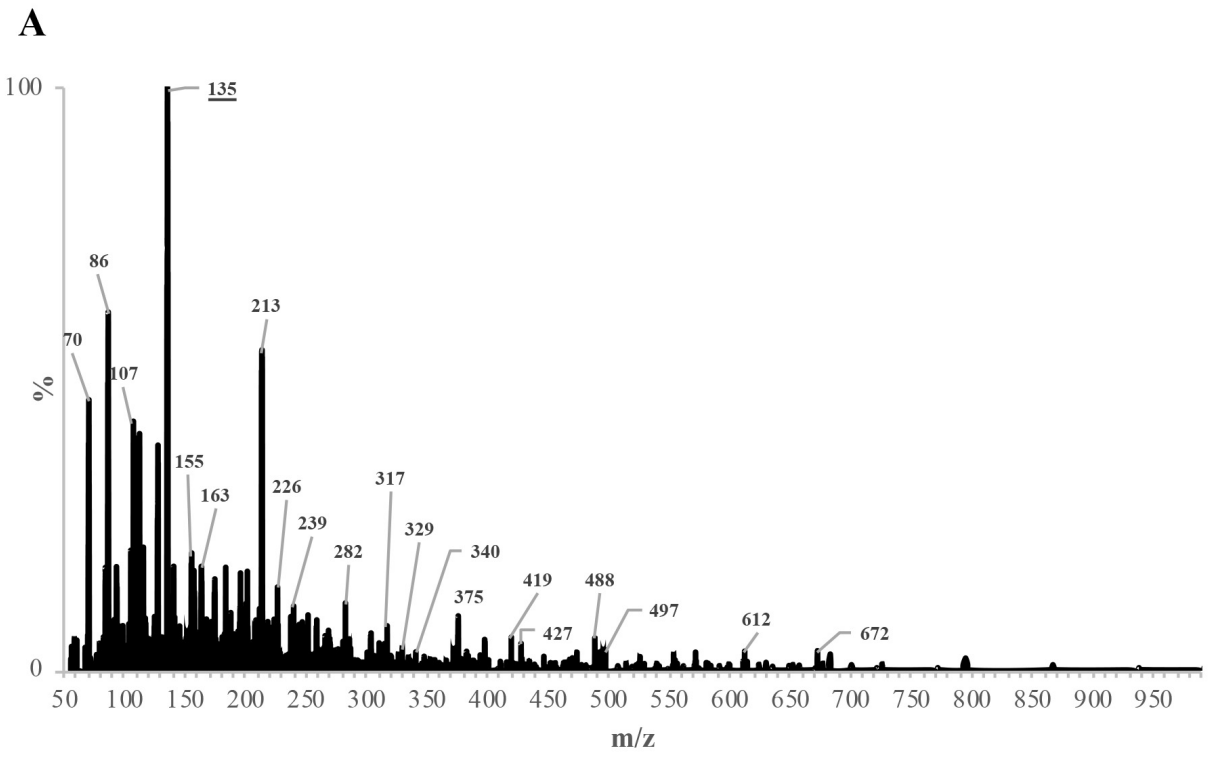
80
60
40
20
0
150
16.6
0
[Benzbromarone, µM]


A
100
%
0
135
86
213
70
107
155
163
226
239
282
317
329
340
375
419
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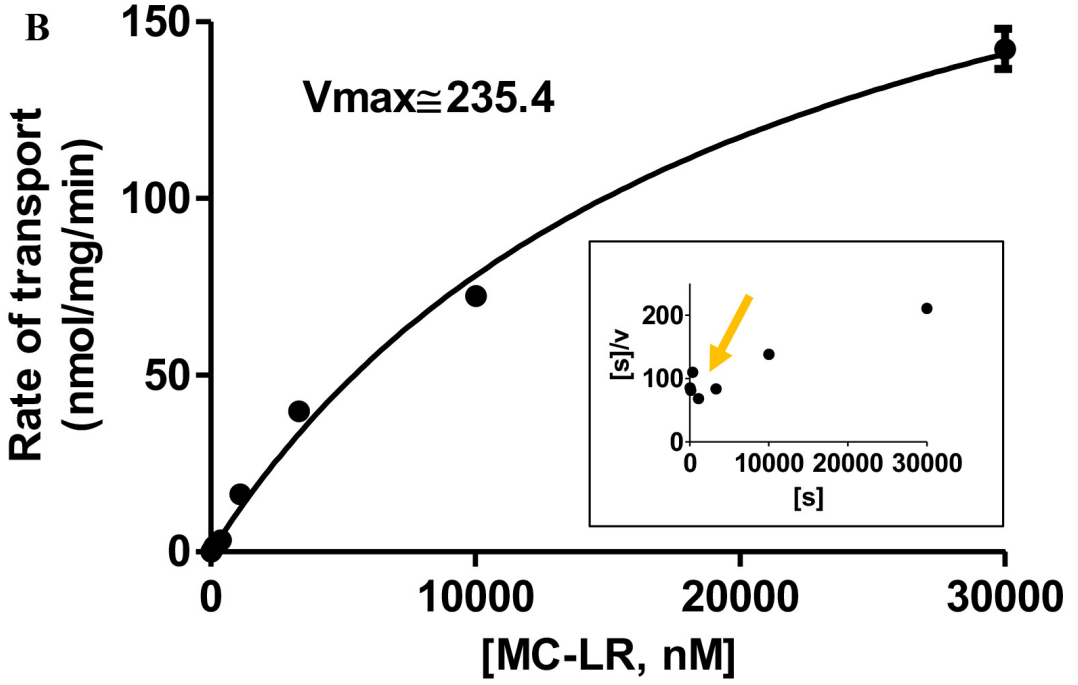
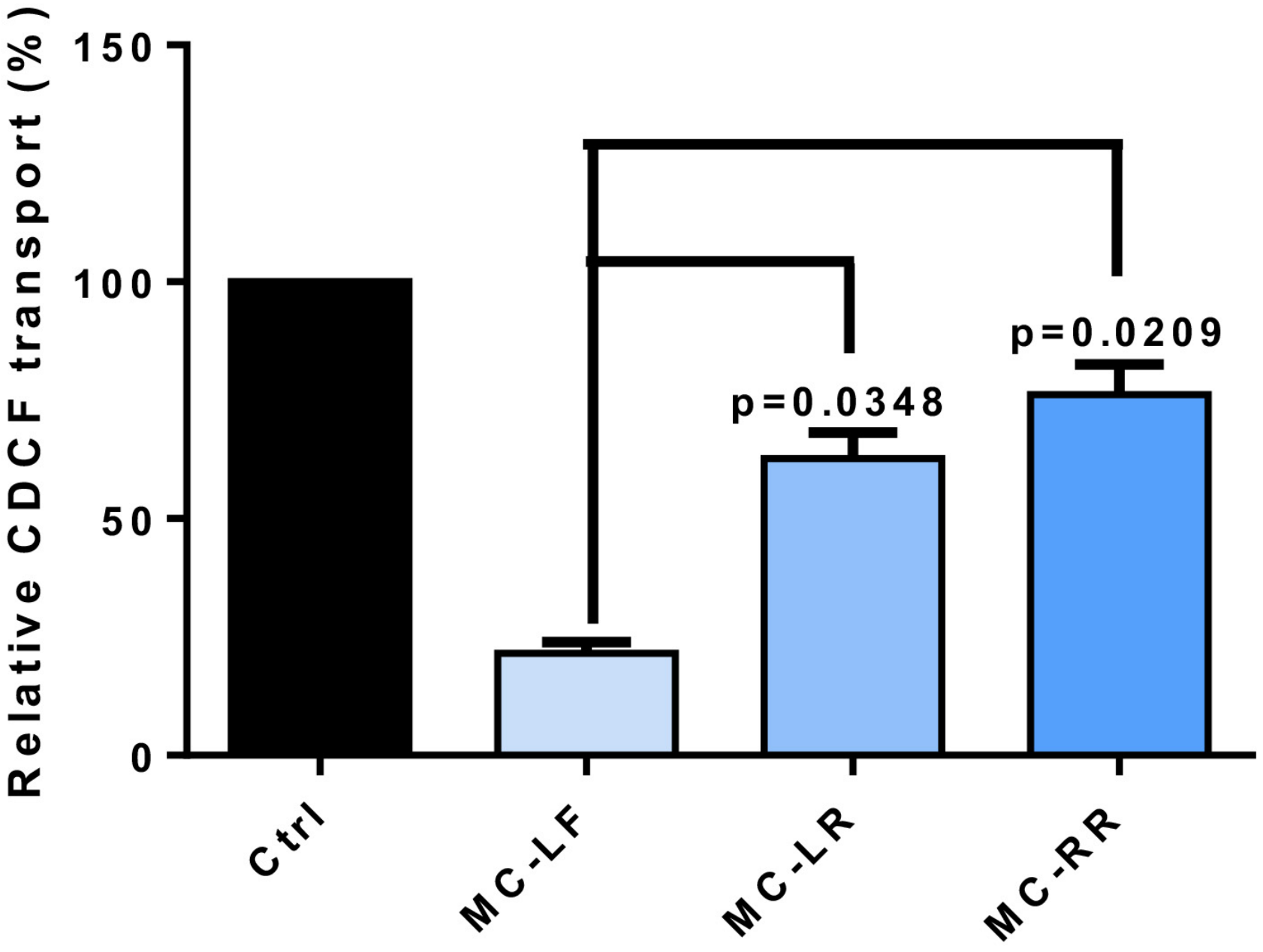
672
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950
m/z


B
Vmax≅235.4
Rate of transport
(nmol/mg/min)
150
100
50
0
0
10000
20000
30000
[MC-LR, nM]
[s]/v
200
100
0
0 10000 20000 30000
[s]


Relative CDCF transport (%)
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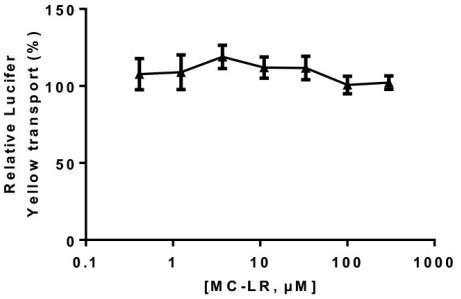
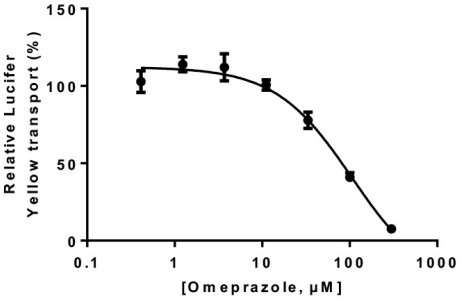
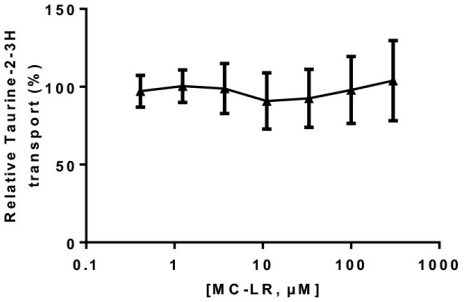
100
50
0
p=0.0348
p=0.0209
Ctrl
MC-LF
MC-LR
MC-RR

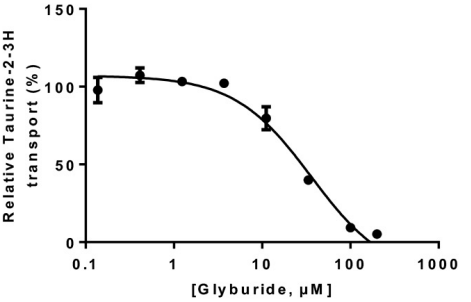


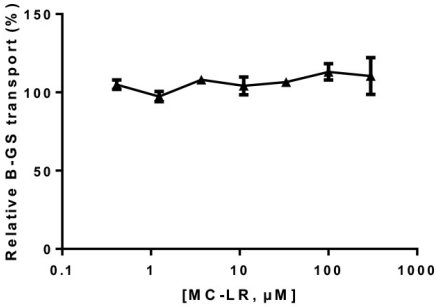
BCRP



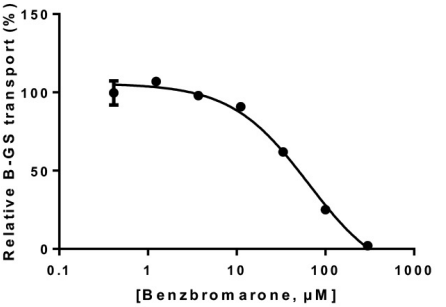


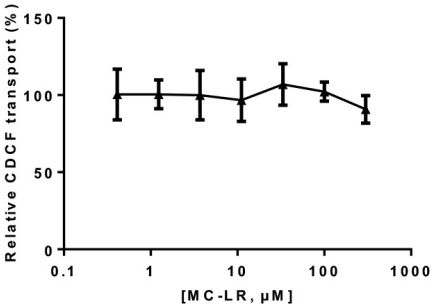
BSEP



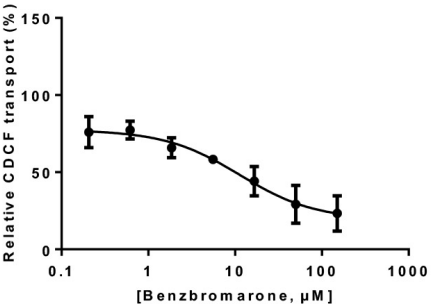


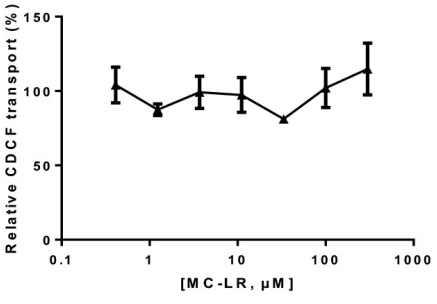
MRP1



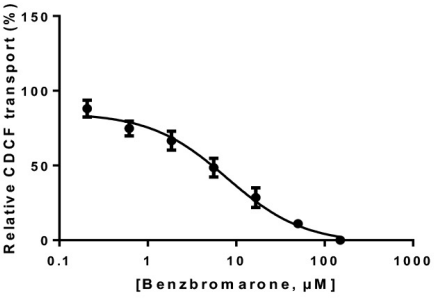


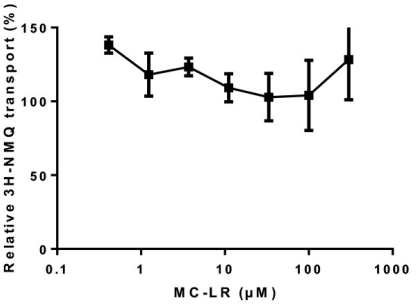
MRP3



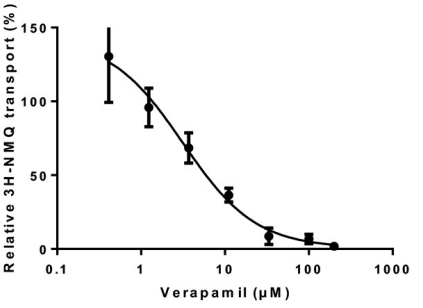


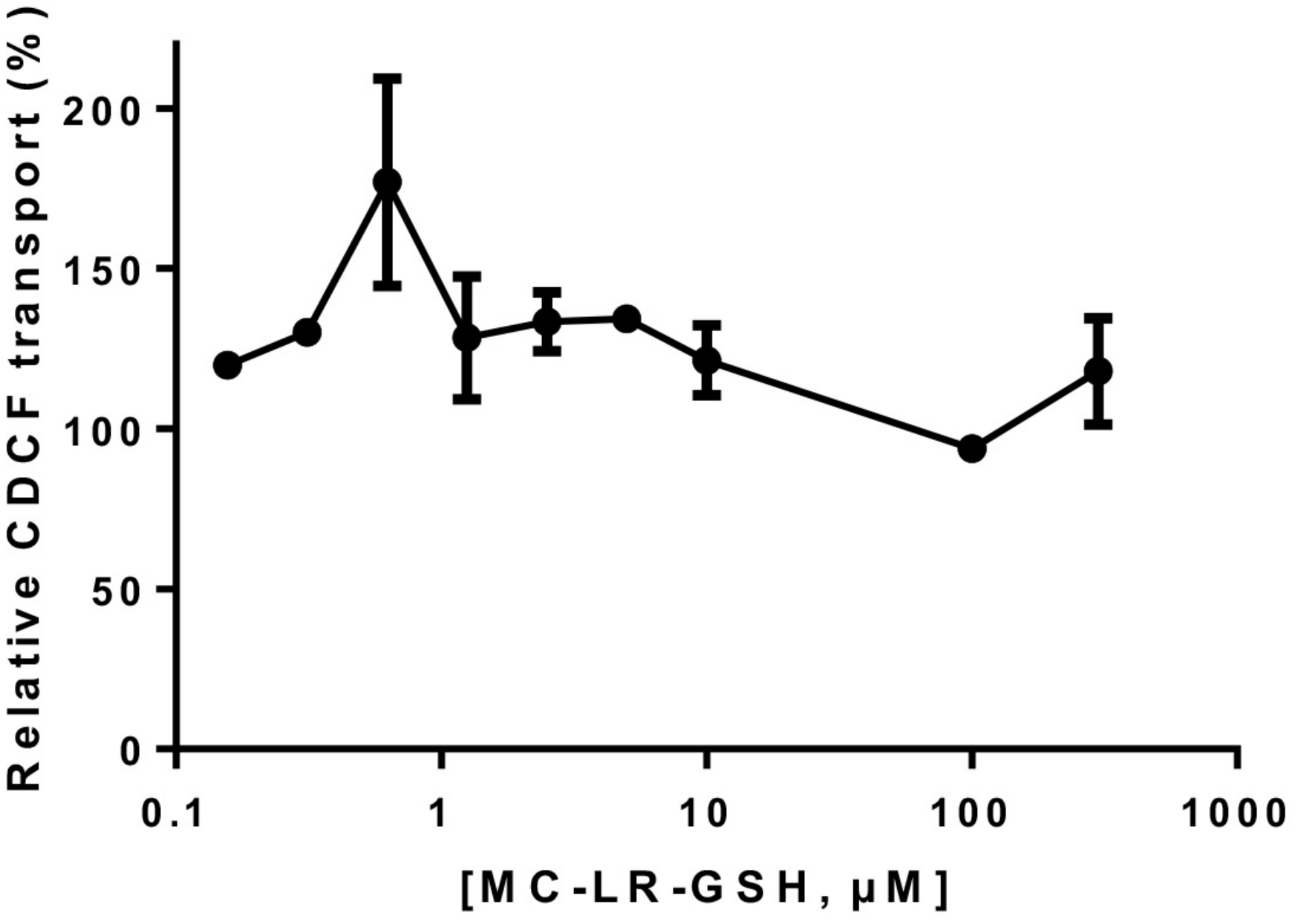
MRP5

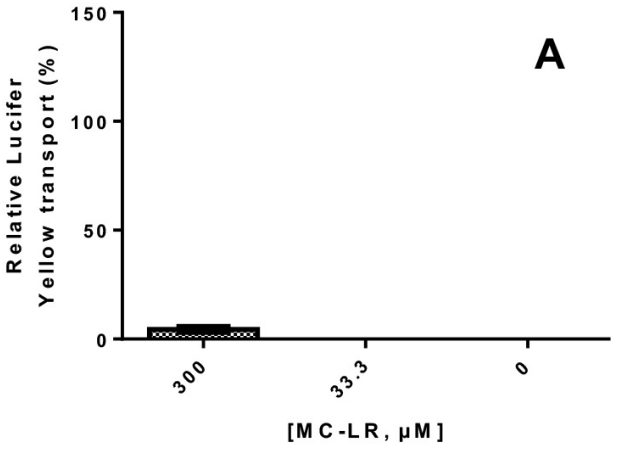


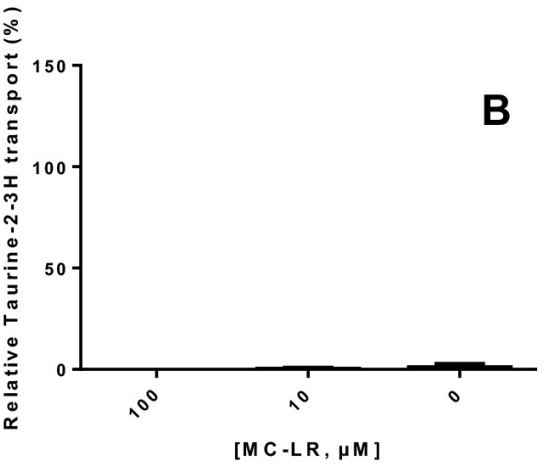


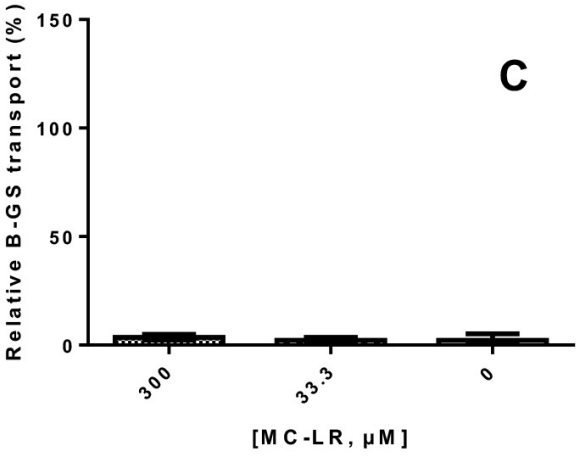
MDR1

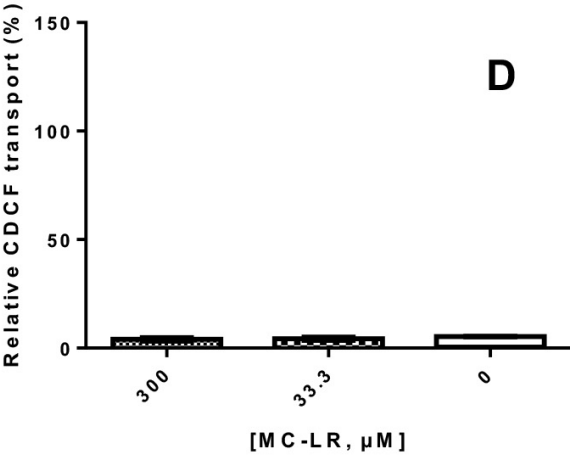


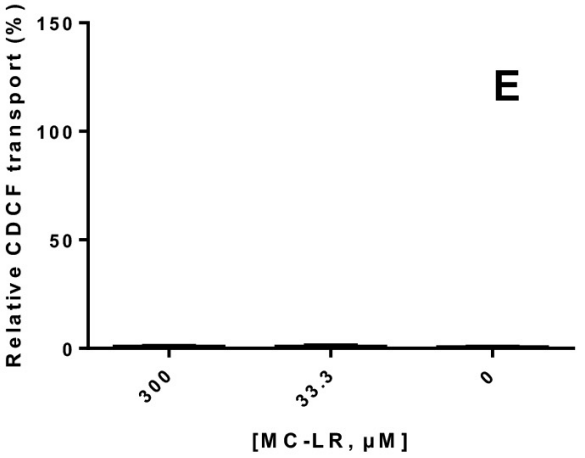

Relative CDCF transport (%)
200
150
100
50
0
0.1
1
10
100
1000
[MC-LR-GSH, µM]

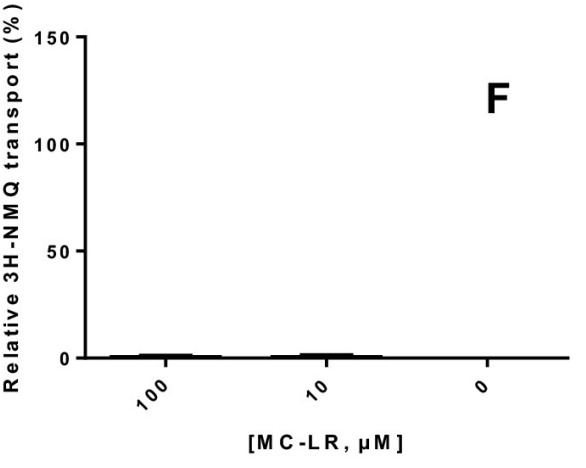

A
Relative Lucifer Yellow transport (%)
150
100
50
0
300
33.3
0
[MC-LR, µM]

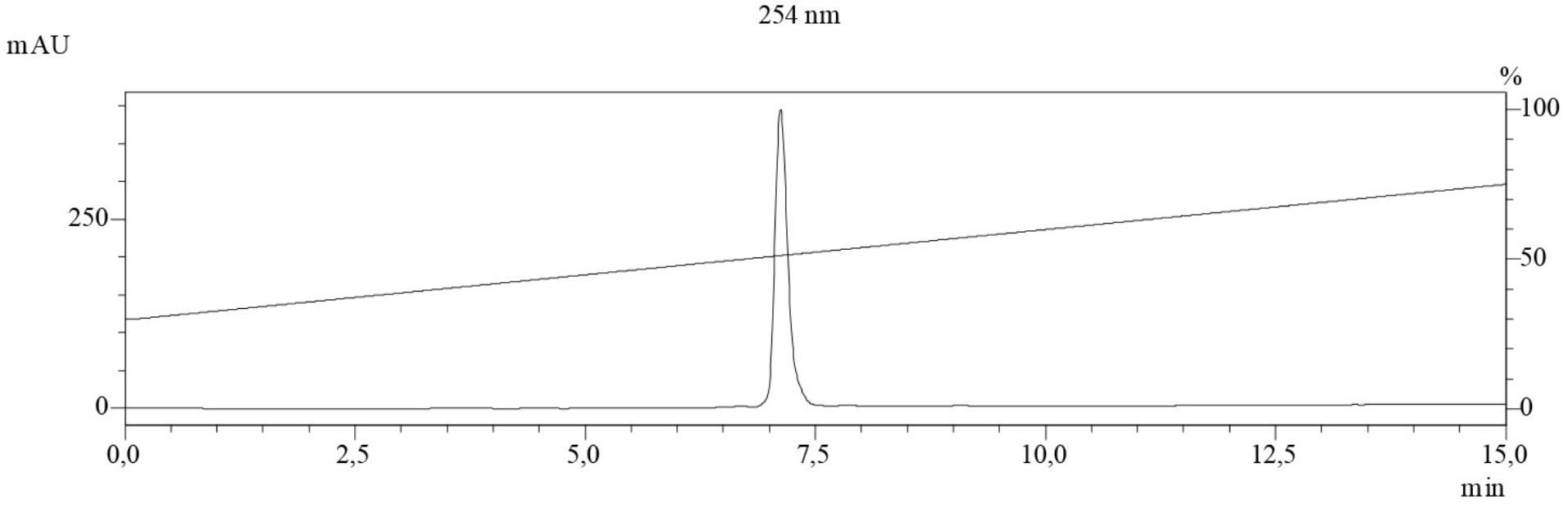

B
Relative Taurine-2-3H transport (%)
150
100
50
0
100
10
0
[MC-LR, µM]


C
Relative B-GS transport (%)
150
100
50
0
300
33.3
0
[MC-LR, µM]


D
Relative CDCF transport (%)
150
100
50
0
300
33.3
0
[MC-LR, µM]


E
Relative CDCF transport (%)
150
100
50
0
300
33.3
0
[MC-LR, µM]


F
Relative 3H-NMQ transport (%)
150
100
50
0
100
10
0
[MC-LR, µM]


254 nm
mAU
%
250
0
100
50
0
0,0
2,5
5,0
7,5
10,0
12,5
15,0
min